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TRENBOLONE CAUSES MORTALITY AND ALTERED SEXUAL DIFFERENTIATION IN *XENOPUS TROPICALIS* DURING LARVAL DEVELOPMENT

ALLEN W. OLMSTEAD,*† PATRICIA A. KOSIAN,† RODNEY JOHNSON,† PAMELA E. BLACKSHEAR,‡ JONATHAN HASELMAN,† CHAD BLANKSMA,† JOSEPH J. KORTE,† GARY W. HOLCOMBE,† EMILY BURGESS,† ANNELIE LINDBERG-LIVINGSTON,† BLAKE A. BENNETT,† KACIE K. WOODIS,† and SIGMUND J. DEGITZ†

†National Health and Environmental Effects Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, Duluth, Minnesota

‡Integrated Laboratory Systems, Research Triangle Park, North Carolina, USA

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Abstract—Trenbolone is an androgen agonist used in cattle production and has been measured in aquatic systems associated with concentrated animal-feeding operations. In this study, the authors characterized the effects of aqueous exposure to 17β-trenbolone during larval *Xenopus tropicalis* development. Trenbolone exposure resulted in increased mortality of post-Nieuwkoop–Faber stage 58 tadpoles at concentrations ≥100 ng/L. Morphological observations and the timing of this mortality are consistent with hypertrophy of the larynx. Development of nuptial pads, a male secondary sex characteristic, was induced in tadpoles of both sexes at 100 ng/L. Effects on time to complete metamorphosis or body sizes were not observed; however, grow-outs placed in clean media for six weeks were significantly smaller in body size at 78 ng/L. Effects on sex ratios were equivocal, with the first experiment showing a significant shift in sex ratio toward males at 78 ng/L. In the second experiment, no significant effects were observed up to 100 ng/L, although overall sex ratios were similar. Histological assessment of gonads at metamorphosis showed half with normal male phenotypes and half that possessed a mixed-sex phenotype at 100 ng/L. Hypertrophy of the Wolffian ducts was also observed at this concentration. These results indicate that larval 17β-trenbolone exposure results in effects down to 78 ng/L, illustrating potential effects from exposure to androgenic compounds in anurans. Environ. Toxicol. Chem. 2012;31:2391–2398. © 2012 SETAC

Keywords—Amphibians Endocrine disruptors Androgens Hormone mimics

INTRODUCTION

Trenbolone is a synthetic androgen commonly used in the cattle industry, where it is typically administered to cattle in the form of trenbolone acetate via subcutaneous implants. Trenbolone acetate is hydrolyzed in the cattle to its biologically active forms, 17β-trenbolone and 17α-trenbolone, with further metabolism resulting in trendione [1]. 17α-Trenbolone is active as an androgen receptor agonist; however, its potency was about 20 times less than that of 17β-trenbolone when examined with recombinant human receptors [2] and about 30 times less potent when examined in an MDA-kb2 cell line assay [3]. Results from in vivo assays with the fathead minnow (*Pimephales promelas*), however, suggest that 17α-trenbolone has activity similar to that of 17β-trenbolone in intact organisms, possibly because of internal conversion of 17α-trenbolone to 17β-trenbolone [4]. These metabolites are detectable in solid dung and liquid manure from implanted cattle [5]. This results in a pathway for trenbolone to enter into the environment, with detectable levels of trenbolone having been measured downstream from cattle feedlots in the low nanograms per liter range [6].

The effects of trenbolone exposure on fish have been studied with respect to physiological processes regulated via the hypothalamus–pituitary–gonad axis, mainly relating to gonad differentiation and development. Researchers in the field of aquaculture, in which males of a particular species have greater economic value, have demonstrated that dosing larval fish with trenbolone results in monosex, male-only cultures of channel catfish (*Ictalurus punctatus*) [7], blue tilapia (*Oreochromis aureus*) [8], black crappie (*Pomoxis nigromaculatus*) [9], and guppies (*Poecilia reticulata*) [10]. Similarly, research in environmental toxicology has demonstrated that aqueous exposure to trenbolone results in complete sex reversal of females in which all male cohorts were produced in zebrafish (*Danio rerio*) [11–13]. Ovotestes characterized by ovaries that developed tubules containing spermatocytes were induced in mosquitofish (*Gambusia affinis*) exposed to trenbolone [14]. Females exposed to trenbolone have been reported to acquire male secondary sex characteristics such as tubercles in adult fathead minnows (*Pimephales promelas*) [15] and papillary processes in Japanese medaka (*Oryzias latipes*) [16]. Trenbolone exposure has also been reported to reduce circulating estradiol and testosterone levels in fathead minnows [15]. Decreased circulating vitellogenin in females has been demonstrated in several fish species exposed to this toxicant [14–16].

To date, no studies have reported the effects of trenbolone exposure in an amphibian species. Although studies exposing larval amphibians to testosterone and other androgens have been described, most focus on the effects on gonad differentiation. Results from such studies vary across species, with some subjects being unaffected and others masculinized [17,18]. For example, many *Rana* species undergo masculinization of the gonads during larval exposure to androgenic compounds [17]. In *Xenopus laevis*, larval exposure resulted in equal ratios of males to females with respect to the gonad

All Supplemental Data may be found in the online version of this article.

* To whom correspondence may be addressed (allen.olmstead@bayer.com).

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phenotype; however, females developed male secondary sex characteristics [19]. For the same species, Gallien [20] reported that testosterone exposure resulted in intersexed specimens.

The goal of these studies was to characterize the toxicity of 17β-trenbolone to developing tadpoles of the western tropical clawed frog (*Xenopus tropicalis*). Exposures to these chemicals were carried out from just after fertilization of the eggs until metamorphosis was complete, at which point sexual differentiation of the gonads and expression of secondary sex characteristics were assessed. These results provided data relevant for risk assessment of this specific chemical and identified the potential array of effects in anurans that might occur with exposure to environmental chemicals possessing androgen agonist activity.

METHODS AND MATERIALS

Animal care and culture

Xenopus tropicalis, "golden" strain, was obtained originally from the laboratory of Richard Harland at the University of California at Berkeley. All frogs were housed in 7-L glass aquaria in a flow-through system using filtered water from nearby Lake Superior at a flow rate of 25 ml/min. Frogs were kept at 25°C with a 12:12-h light:dark photoperiod. Adult frogs were fed premium sinking frog food pellets (Xenopus Express). Breeding pairs were induced to mate by injections of human chorionic gonadotrophin (Sigma-Aldrich). Both males and females receive a priming injection of 20 IU gonadotrophin, followed by a boosting injection of 100 IU gonadotropin 6 to 7 h after the priming injection. Staging of tadpoles was performed by using Nieuwkoop–Faber (NF) staging guidelines [21]. Tadpoles were fed a diet of carrot, spinach, Sera Micron (Sera North America), and 24-h-old brine shrimp nauplii.

First trenbolone exposure

17β-Trenbolone was obtained from Sigma-Aldrich with a reported purity of >98% (CAS 10161-33-8). Exposures to nominal concentrations of 78, 310, 1,250, and 5,000 ng/L were performed in an enclosed flow-through diluter system set to a flow rate of 25 ml/min using Lake Superior water. Tank volumes were 4 L, resulting in nine tank turnovers per day. Water quality parameters of temperature, pH, dissolved oxygen, conductivity, alkalinity, and hardness were monitored periodically. Exposures were initiated using 30 tadpoles <24 h old; on the day following mating, developing embryos were washed in 20 g/L cysteine (pH 8.1) for 1 min to remove gel coats. Only normally developing embryos as determined by microscopic examination were used. Each treatment and control was replicated three times in a blocked design. Blocks consisted of three separate spawns, with each spawn represented once within a single treatment tank. Exposure continued until completion of metamorphosis. On each day, tadpoles that had completed metamorphosis (NF stage 66) were removed from the exposure system and were randomly assigned to two groups. One group was immediately sampled on the day on which they completed metamorphosis, whereas the other group was raised in control Lake Superior water until six weeks postmetamorphosis.

Concentrations of 17β-trenbolone were determined weekly in water from each of the treatment tanks except for the lowest treatment (78 ng/L). Water samples (1.5 ml) were placed in amber vials and immediately analyzed with an Agilent 1100 series high-performance liquid chromatograph (HPLC) with fluorescence detection. The excitation and emission wavelengths were 364 nm and 460 nm, respectively. An aliquot (500 μl) of sample was loaded onto a Synergi Hydro RP, 4-μm, 150- × 4.6-mm column (Phenomenex) that was maintained at 35°C. The column was eluted under isocratic conditions with methanol (70%) and water (30%) at a flow rate of 1 ml/min. A Lake Superior water blank, matrix-spiked sample at 1,010 ng/L and a duplicate sample from one of the exposure tanks were analyzed in conjunction with each sample set. No 17β-trenbolone was detected in the Lake Superior water blanks ($n = 11$). Mean ± standard deviation ($n = 11$) recovery of spiked samples was 99 ± 2.9%. Agreement among duplicate samples was 100 ± 0.9%. The detection limit was 100 ng/L.

All sampled frogs were euthanized either by submersion in 200 mg/L buffered tricaine methanesulfonate for those frogs sampled at NF stage 66 or by injection of 50 μl of 10% tricaine methanesulfonate in phosphate-buffered saline for frogs sampled at six-weeks postmetamorphosis. Body sizes were assessed as both a wet body weight and snout–vent length. The gonads were assessed morphologically for phenotypic sex under a microscope. For those sampled at completion of metamorphosis, gonads were categorized based on pigmentation and morphological shape. Ovaries at this stage are characterized by having a long, scalloped shape with rows of pigmentation. Testes are distinguished by a complete lack of pigmentation and a smooth profile, with the typical oval shape beginning to form just posterior to the fat body. These tissues are translucent at this stage of development, and a drop of fixative was added to the gonads to aid in visualization. For six-week, postmetamorphic frogs, male testes were paired, small, white, kidney-bean-shaped organs located adjacent and posterior to the fat body. Female gonads were paired, lobed organs immediately medial to the kidneys.

Second trenbolone exposure

A second exposure using 17β-trenbolone was performed similarly to that described above, with any differences described below. For this exposure, nominal concentrations were 3.7, 11, 33, and 100 ng/L. Tadpoles were allowed to hatch and were approximately 48 h old when we initiated exposure, at which point they had just become free swimming. Replication consisted of eight tanks for controls and five tanks for each of the exposure concentrations. Exposure continued until metamorphosis was complete (NF stage 66), with tanks examined daily for tadpoles that had reached this stage. These were removed from the exposure system each day and randomly assigned to two groups. One group was immediately sampled on the day they completed metamorphosis, whereas the other group was placed in tanks that contained control Lake Superior water. Only results for animals sampled at completion of metamorphosis were included in this study.

All sampled frogs were euthanized by immersion in tricaine methanesulfonate as described above. Body length, body weight, and phenotypic sex were assessed similarly to the first experiment. Additionally, forelimbs of newly metamorphosed individuals were assessed for the development of nuptial pads, which are male secondary sex characteristics [22]. Each individual was scored based on the presence or absence of pigmented epidermal spines on the undersides of the forelimbs with the aid of a microscope. In situ kidney–gonad complexes from each frog were fixed in modified Davidson's fixative for 48 h and then stored in 10% neutral buffered formalin. Kidney–gonad complexes were assessed histologically with 4-μm-thick transverse step sections selected at 100-μm intervals. Staining used hematoxylin and eosin. All histological processing was performed at Integrated Laboratory Systems. In total 35, 20, 19,

22, and 17 specimens were assessed by histology from the control at 3.7, 11, 33, and 100 ng/L concentrations, respectively.

Weekly water samples were collected from the 17β-trenbolone exposure tanks and placed into clean amber bottles. Sample volumes were adjusted to 100 ml with clean Lake Superior water and immediately processed through solid-phase extraction (SPE) columns using a vacuum manifold. Briefly, 3 ml (200 mg) Strata X C-18 SPE columns (Phenomenex) were precleaned with 5 ml acetronitrile, activated with 10 ml methanol, and then equilibrated with 10 ml Burdick & Jackson HPLC-grade water (B&J water). Samples were processed through the columns under vacuum (5 mm Hg) and rinsed with 2 ml B&J water and dried under vacuum (15 mm Hg) for 3 min. 17β-Trenbolone was slowly eluted from the columns, under low vacuum (1 mm Hg), with four 1 ml aliquots of methanol. Eluates were evaporated to dryness with nitrogen and a heated water bath (40°C) and reconstituted to a known volume with 10% methanol and 90% B&J water. Quality assurance samples were conducted with each SPE sample set and consisted of a Lake Superior water blank, as well as duplicate and matrix-spiked samples at each of the 17β-trenbolone treatment levels.

17β-Trenbolone concentrations were determined by reverse-phase liquid chromatography with a triple quadrupole mass spectrometer (LC-MS/MS). Aliquots of the SPE-reconstituted extracts and an internal standard, allyl trenbolone, were placed into amber HPLC vials. The samples were then analyzed with an Agilent 1200 HPLC with a 6410 triple quadrupole mass spectrometer. Each sample (50 μl) was loaded onto an Agilent Zorbax Extend C-18 column (3.5-μm particle, 2.1 × 100 mm) maintained at 30°C. The column was eluted under isocratic conditions at a flow rate of 0.2 ml/min using a mobile phase consisting of 2% ethanol:67% methanol:31% ammonium acetate (2.5 mM):0.0025% concentrated ammonium hydroxide (v/v). The HPLC effluent was introduced into the mass spectrometer by means of electrospray ionization in the positive mode using nitrogen as the nebulizing and collision gas. Multiple reaction-monitoring parameters were collision energy (20 V), fragmentor (150 V), precursor ion (271 *m/z*), quantifier ion (199 *m/z*), and qualifier ion (157 *m/z*) for 17β-trenbolone and collision energy (22 V), fragmentor (150 V), precursor ion (311 *m/z*), quantifier ion (227 *m/z*), and qualifier ion (199 *m/z*) for the internal standard. No 17β-trenbolone was detected in the Lake Superior water blanks ($n = 10$). Mean ± standard deviation ($n = 10$) recoveries of spiked samples were 64 ± 7, 65 ± 5, 69 ± 6, and 71 ± 8% at the 3.7, 11, 33, and 100 ng/L spiked levels, respectively. Mean ± standard deviation ($n = 8$) agreement among duplicate samples were 101 ± 5, 97 ± 5, 97 ± 6, and 97 ± 2% for the 3.7, 11, 33, and 100 ng/L treatments, respectively. The method limit of quantification was 1.5 ng/L.

Statistical analysis

Measurements of individual tadpoles in each tank were averaged, and these averages were used in statistical analyses, making the experimental unit the tank. For the time-to-metamorphosis endpoint, median metamorph times were calculated for individual tanks using Kaplan–Meier estimates in R software [23]. For the first exposure, differences in survivorship were analyzed using a Wilcoxon nonparametric comparison of the control to the treatment groups [24]. For all other endpoints in the first exposure, data from the control and lowest treatment were analyzed using a paired *t* test with separate spawns as blocks [24]. For the second exposure, differences from control tanks were evaluated using a generalized linear model with Dunnett's *t* test with separate spawns as blocks (R software). Sex ratios from the second exposure were arcsine transformed before statistical analysis. Histological data were analyzed using a log-likelihood ratio (G test) for contingency tables comparing the four treatments separately with the controls with a Bonferroni correction to account for multiple comparisons. Analyses of body mass and length were conducted regardless of sex, because sexual dimorphisms in this species do not occur until after 10 weeks of postmetamorphic development [22].

RESULTS

Trenbolone concentrations

For the first exposure, mean ± standard deviation ($n = 30$) measured concentrations of the 310, 1,250, and 5,000 ng/L 17β-trenbolone treatments were 280 ± 40, 1,060 ± 160, and 4,200 ± 800 ng/L, respectively, over the duration of the experiment. Among-tank variability in a given treatment was generally ≤20%, with coefficients of variation (averaged across the duration of the exposure) of 13, 15, and 20% for the 310, 1,250, and 5,000 ng/L treatments, respectively. No 17β-trenbolone was detected in the control water. Trenbolone measurements over time are depicted in Supplemental Data, Figure S1.

For the second exposure, mean ± standard deviation ($n = 25$) measured concentrations of the 3.7, 11, 33, and 100 ng/L 17β-trenbolone treatments were 3.4 ± 0.9, 11 ± 2, 32 ± 6, and 102 ± 19 ng/L, respectively, over the duration of the experiment. Among-tank variability was ≤25%, with the coefficient of variation (averaged across the duration of the exposure) of 25, 21, 19, and 19% for the 3.7, 11, 33, and 100 ng/L treatments, respectively. No 17β-trenbolone was detected in the control water treatments. Trenbolone measurements over time are depicted in Supplemental Data, Figure S1.

Mortality

In the first trenbolone exposure, almost all tadpoles died with the 310 and 1,250 ng/L treatments (Table 1 and Supplemental Data, Fig. S2). The average control mortality, which occurred primarily during the first 3 d, was 11%. Mortality at the 78 ng/L treatment was not significantly different from the control. The mortalities at the higher treatments occurred after the tadpoles had reached NF stage 58 and presented consistent morphological characteristics (Fig. 1). In these late-stage mortalities, the tadpoles would be found floating upside down in the tanks. Necropsies of these animals revealed the presence of air inside the stomach and intestinal tract along with uninflated lungs. During the six-week grow-out period, three frogs from the control tanks and three frogs from the 78 ng/L tanks died.

On day 43, all remaining tadpoles in the 5,000 ng/L trenbolone treatment were moved to chemical-free Lake Superior water in a deviation from the experimental protocol. At this point, roughly 30% of the tadpoles in this treatment had died. Among the tadpoles that were moved, 49 ± 5% survived to metamorphosis. Those that died were those that were more developed, i.e., closer to NF stage 58, at the time of the move. The surviving tadpoles tended to be less well developed than those that died. Because these individuals were removed from the experiment before the end of the exposure, they were excluded from any statistical analysis that included the other treatment levels.

In the second exposure, significant mortality occurred with the highest treatment (100 ng/L) in a manner consistent with that observed in the first exposure (Table 2 and Supplemental Data, Fig. S2). Lower mortality was experienced when the

Table 1. In the first trenbolone exposure, significant differences were observed in survivorship, body size of postmetamorphic frogs, and sex ratios of *Xenopus tropicalis*[a]

Endpoint	Control	78 ng/L	310 ng/L	1,250 ng/L
Larval survival (%)	90.8 ± 1.6	82.0 ± 9.3	5.6 ± 6.9*	0.0 ± 0.0*
Median time to metamorphosis (d)	50.3 ± 0.6	52.5 ± 2.5	NA	NA
Body mass at metamorphosis (g)	0.74 ± 0.03	0.77 ± 0.08	NA	NA
Body length at metamorphosis (mm)	17.4 ± 0.1	17.4 ± 0.5	NA	NA
Body mass at six weeks postmetamorphosis (g)	6.0 ± 0.5	4.3 ± 0.7*	NA	NA
Body length at six weeks postmetamorphosis (mm)	35.6 ± 1.4	31.5 ± 2.6*	NA	NA
Sex ratio (% male)	52 ± 4	75 ± 8*	NA	NA

[a] Data are presented as the average across tanks ($n = 3$) in a treatment ± standard deviation. Because of the removal of the 5,000 ng/L treatment level midway through the experiment, data collected from this level are not included in any analyses.
* Significant difference from the control ($\alpha = 0.05$).
NA = not applicable.

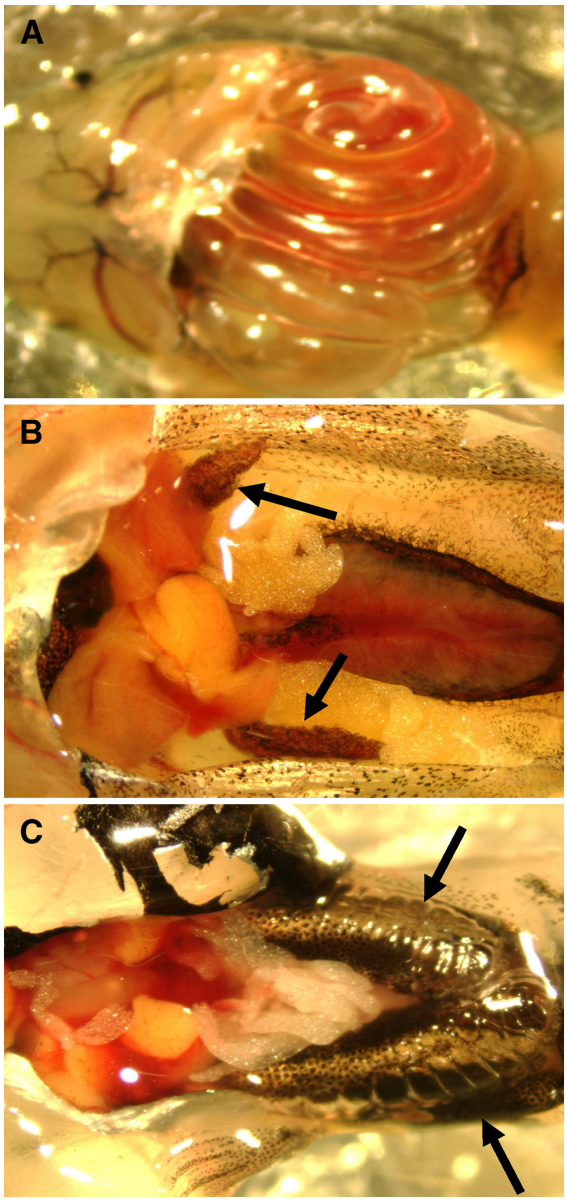

Fig. 1. Trenbolone exposure during larval development caused mortality with a specific presentation. Significant mortality occurred in both exposures at trenbolone concentrations of 100 ng/L and higher. (**A**) This tadpole, sampled from the 310 ng/L treatment, demonstrates an extreme presentation of most trenbolone-induced mortalities. The entire intestinal tract is full of air. (**B**) The same individual as in A, except the intestinal tract has been removed. Arrows point to deflated lungs devoid of air. (**C**) Normal presentation of lungs (arrows) in an NF stage 58 tadpole. [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com]

second exposure was initiated, which is attributed to starting with older, more developed tadpoles.

Developmental effects

No significant effects on time to reach metamorphosis or body size were detected in tadpoles exposed to trenbolone (Table 1 and Supplemental Data, Fig. S3). Both body mass and length, however, were significantly reduced compared with controls in animals from the first exposure that had been raised in control water for six weeks after metamorphosis. This indicates that, although trenbolone effects on growth were not evident at metamorphosis, reduced growth in juveniles was observed, even after chemical exposure ceased.

Pigmented, epidermal spines on the forelimbs were observed in half the tadpoles exposed to 100 ng/L trenbolone (Table 2). These structures are characteristic of nuptial pads, a male secondary sex characteristic. These structures were not observed in tadpoles from the two lower concentration treatments and controls. This is consistent with previous experiments in which these structures were not observed in frogs at this stage of development. Normal development of nuptial pads in males usually begins eight weeks postmetamorphosis, the time at which testosterone can be detected in blood [22]. Some individuals were observed in the 33 ng/L trenbolone treatment with developing nuptial pads (16%), but this observation was not statistically significant within the confines of the experiment. The lack of this observation in historical data suggests, however, that this is biologically significant.

Sex ratios

Larval exposure to 78 ng/L trenbolone in the first exposure resulted in a significantly skewed sex ratio (Table 1). This result was not statistically significant in the second exposure at the 100 ng/L treatment (Table 2), although the percentage of males in this treatment was similar to that observed in the first exposure. This difference between the two studies appears to be strictly statistical in nature, with higher variances being observed in the second exposure.

Histology

Control female gonads were characterized as being in either stage III or stage IV as Ogielska et al. [25] described. In general, these ovaries were characterized as possessing a cortex containing primary or secondary oogonia and a central lumen (Fig. 2). In control males, testes contained nests of spermatogonia and primary spermatocytes separated by spindle-shaped pre-Sertoli cells. An intersex gonad phenotype was observed in the highest treatment level. This was characterized by a medullary region with primary germ cells and a cortical region with

Table 2. In the second trenbolone exposure, significant effects were observed with the highest treatment level compared with controls for larval survival and the development of nuptial pads in *Xenopus tropicalis* tadpoles[a]

Endpoint	Control	3.7 ng/L	11 ng/L	33 ng/L	100 ng/L
Larval survival (%)	89.1 ± 5.5	88.0 ± 5.0	92.0 ± 1.8	82.4 ± 11.0	59.3 ± 15.5*
Median time to metamorphosis (d)	51.2 ± 2.5	53.0 ± 4.5	54.4 ± 4.1	49.0 ± 1.6	51.6 ± 6.0
Body mass (g)	0.67 ± 0.06	0.66 ± 0.08	0.64 ± 0.13	0.65 ± 0.07	0.73 ± 0.12
Body length (mm)	16.5 ± 0.3	16.4 ± 0.6	16.1 ± 1.0	16.4 ± 0.8	17.3 ± 1.3
Nuptial pad prevalence (%)	0 ± 0	0 ± 0	0 ± 0	16 ± 26	50 ± 33*
Sex ratio (% male)	58 ± 11	52 ± 20	71 ± 6	58 ± 12	72 ± 17

[a] Data are presented as the average across tanks in a treatment ± standard deviation ($n = 8$ for controls and $n = 5$ for treatments).
* Significant difference from the control ($\alpha = 0.05$).

primary and secondary germ cells (Fig. 2). No treatment-related effects on kidney histology were observed.

Hypertrophy of the Wolffian ducts was observed at the highest treatment level (Fig. 3). In control animals, Wolffian ducts were present within the lateral margins of the kidneys and were characterized by a single layer of low cuboidal epithelial cells. Hypertrophy of these structures was characterized by a gradual change in the height and thickness of the epithelial cells around the circumference of the duct. Ducts were scored as possessing minimal hypertrophy, when unilaterally the duct epithelial cells were up to twice the normal height, with frequent piling or crowding of the nuclei. Mild hypertrophy was scored based on bilateral increases in cell height up to three times normal, with frequent piling and crowding of the nuclei. In moderate to severe legions, the epithelial thickness was greater than three times the thickness of the epithelia in the control specimens and varied from columnar single-nucleated cells to tall columnar cells with stratified nuclei. Moderate and severe scores were differentiated based on the presence of hypertrophic cells in one level and multiple levels, respectively. The prevalence of Wolffian duct hypertrophy was significantly elevated in the 100 ng/L treatment. Three of 22 specimens evaluated

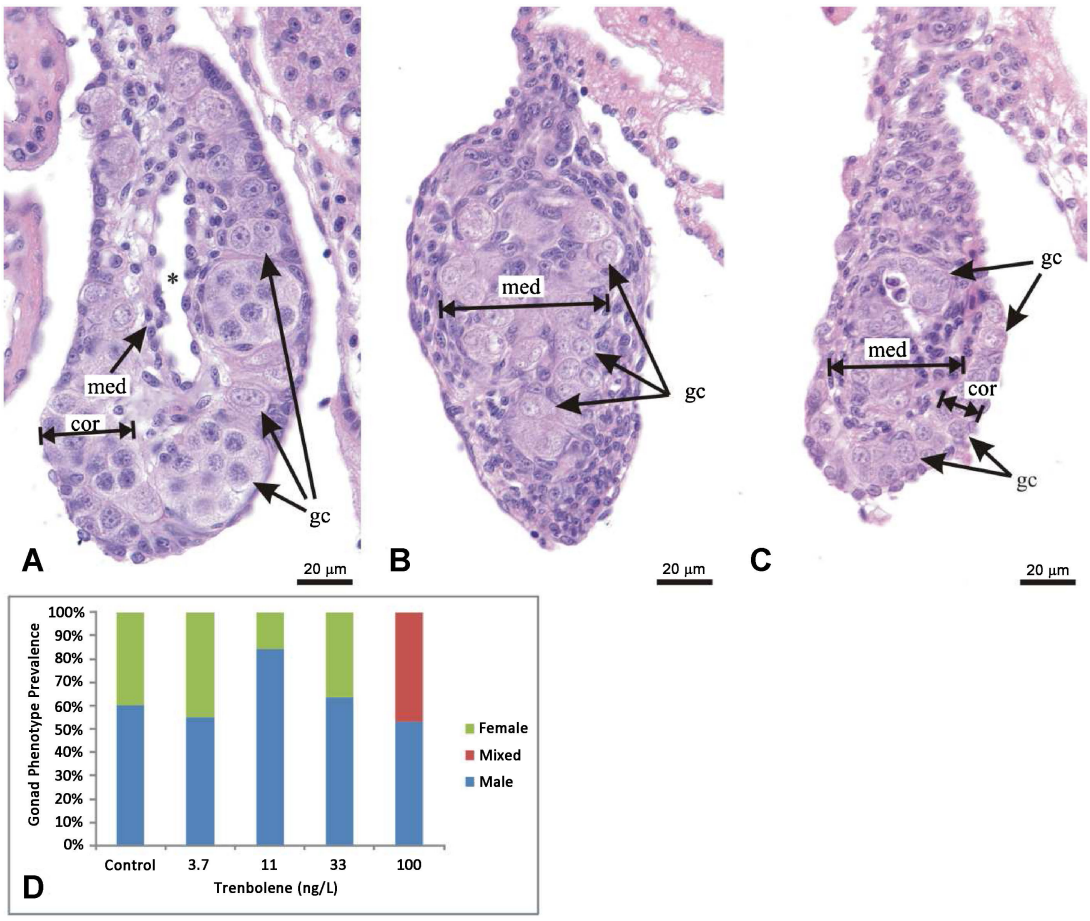

Fig. 2. Trenbolone causes intersex in the 100 ng/L treatment. Gonads from a random subsample of NF66 tadpoles were evaluated histologically for effects on gonad development. A control ovary (**A**) and control testis (**B**) are pictured along with an example of a mixed-sex gonad (**C**). The control ovary (**A**) has a lumen (asterisk) surrounded by a thin layer of medullary cells (arrow) and a fairly thick cortex (cor) filled primarily with germ cells (gc) in various stages of development. The cortex of the control testis (**B**) is composed primarily of small somatic cells, and the medulla (med) contains the germs cells (gc). The intersex gonad has both cortical (cor) and medullary (med) germ cells (gc). Proportions of each phenotype as determined histologically were significantly different from the control in the 100 ng/L treatment (**D**) log likelihood ratio test, $\alpha = 0.05$). For the controls, 35 frogs were examined, and 17 to 22 frogs were examined for treatments. Scale bars = 20 µm. [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com]

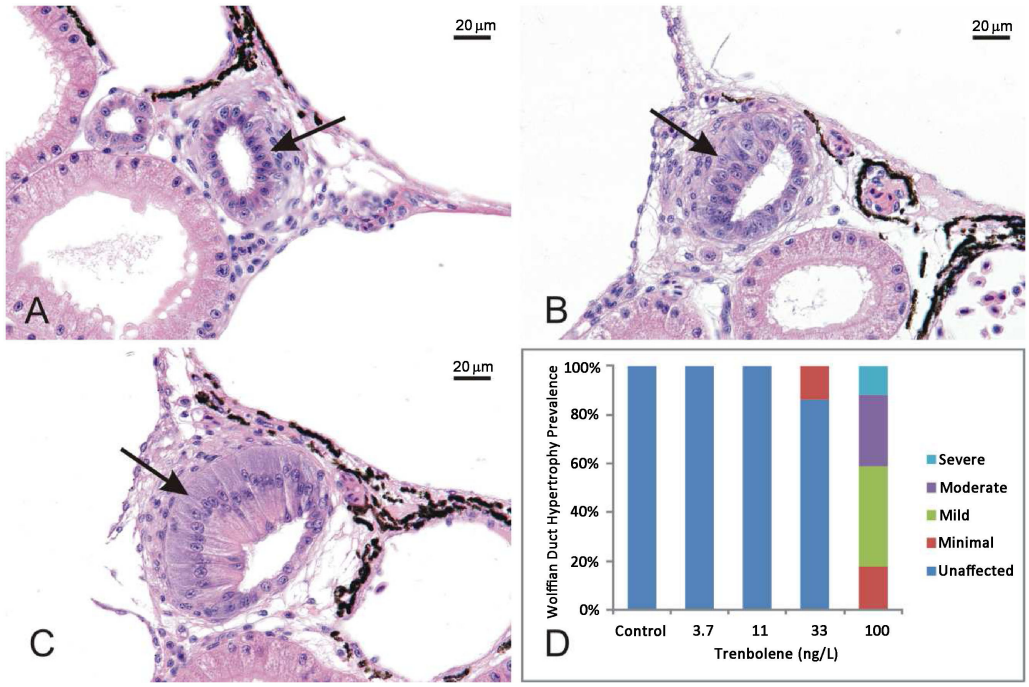

Fig. 3. Trenbolone causes cellular hypertropy in the Wolffian duct epithelium. The epithelial cells in the Wolffian duct of a control animal (**A**) are made up of a single layer of low cuboidal cells (arrow). Mild hypertrophy shown in (**B**) is characterized by asymmetric thickening of epithelial cells to approximately three times that of controls (arrow). Severe hypertrophy (**C**) is characterized by an asymmetric increase in the epithelial cell height greater than three times the control thickness (arrow). (**B**) and (**C**) were sampled from the 100 ng/L treatment. (**D**) Prevalence of Wolffian duct hypertrophy. For the controls, 35 frogs were examined, and 17 to 22 frogs were examined for treatments. Staining used hematoxylin and eosin. Scale bars = 20 µm. [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com]

histologically in the 33 ng/L treatment presented minimal duct hypertrophy, whereas all the specimens from the 100 ng/L treatment had some degree of duct hypertrophy ranging in severity from minimal to severe (i.e., four of 17 minimal, six of 17 mild, five of 17 moderate, and two of 17 severe; see Fig. 3).

DISCUSSION

Exposure to 17β-trenbolone resulted in increased mortality in tadpoles, with a lowest observed effect concentration (LOEC) of 100 ng/L. This mortality occurred late in tadpole development around metamorphic climax. Robertson et al. [26] report that in an aqueous exposure to 30 µg/L testosterone propionate of *Xenopus laevis* starting at NF stage 48, tadpoles develop normally until reaching NF stage 59 or 60. After attaining this stage of development, the tadpoles became moribund, and all had died by NF stage 62. The authors report that these exposed tadpoles showed hypertrophy of the larynx compared with controls and hypothesized that this resulted in mortality by interfering with cardiac or respiratory function. The larynx in this species is sexually dimorphic, becoming masculinized as a result of secreted androgens from the testes during postmetamorphic juvenile development [27]. Our observations of trenbolone-induced mortality in *X. tropicalis* are consistent with this hypothesis [26]. Once the larynxes in these tadpoles become sensitive to androgen stimulation during thyroid hormone-directed metamorphosis, muscular hypertrophy results in the air passageway to the lungs being restricted. Buccal pressure during respiration of the tadpoles forces air through the path of least resistance, that is, the digestive tract instead of the lungs. This results in uninflated lungs and air within the digestive tract, which ultimately kills the organism.

This proposed mechanism of trenbolone-inducted mortality is unique to anuran species. Trenbolone mortality in fish species from trenbolone exposure is not well characterized, because adverse effects, such as those on gonad development and reproduction, are reported at very low concentrations (i.e., about 10–50 ng/L [11–13,15]). In a 60-d larval exposure of zebrafish to 17β-trenbolone, for example, Holbech et al. [12] reported no significant increases in mortality even at concentrations up to 2,800 ng/L.

Nuptial pads in male anurans are characterized by darkly pigmented epidermal spines and modified, subdermal mucous glands on the undersides of the forelimbs and digits [28]. In male *X. tropicalis*, these structures appear about eight-weeks postmetamorphosis, immediately after levels of circulating testosterone become detectable [22]. In adult male *X. laevis*, nuptials pads regress following castration, whereas exposure to androgens in adult females cause the nuptial pads to develop [29]. Chang and Witschi [19] reported that testosterone exposure in larval *X. laevis* resulted in "little toads that all have well developed dark arm and hand pads and immediately start embracing each other" after metamorphosis. Nuptial pads were observed neither in any of the control tadpoles nor at the lowest trenbolone treatments. Higher trenbolone exposures resulted in the development of the epidermal spines regardless of phenotypic sex after metamorphosis was complete. These results demonstrate that nuptial pad development in larval amphibian toxicity assays could serve as a specific, easy, and cost-effective indicator of androgenic activity.

Early gonad differentiation in larval *X. laevis* has been described extensively [21,27] and would be expected to be similar to that of *X. tropicalis*. During gonad differentiation, the primordial germ cells migrate from the mesonephros into the developing gonads, where they are enveloped by epithelial cells. In females, these primordial germ cells migrate to the exterior cortex, and a cavity forms in the center of the interior medulla. Within the ovary, the germ cells become surrounded

by follicular cells and differentiate into oocytes. In males, by contrast, primordial germ cells migrate into the medulla region where seminiferous tubules develop, and the germ cells differentiate into spermatogonia. In the 100 ng/L trenbolone treatment, gonads were either testis or intersex. This suggests that, with this treatment, gonad differentiation in female tadpoles was altered by trenbolone, changing the presumptive ovary into an intersex phenotype. The gross morphological characterization of the gonads in both experiments was not sensitive to detection of this mixed-sex phenotype; the affected individuals appeared to possess a female phenotype. The significant increase in males with respect to sex ratios in the first exposure is consistent with trenbolone masculinizing female gonads and is consistent with the histopathological findings from the second exposure. These results are also similar to those of a previous study conducted with methyl testosterone and *X. laevis* in which exposed tadpoles exhibited a significant male bias in sex ratio [30].

Wolffian ducts in amphibians function as urinary ducts in both males and females, but in males they also function as sperm ducts or vasa deferentia [31]. The hypertrophy of the epithelial cells lining the Wolffian ducts of trenbolone-exposed *X. tropicalis* observed here is consistent with previous studies using larval tiger salamanders (*Ambystoma tigrinum*). Larval salamanders injected with dihydrotestosterone displayed increased cross-sectional area of the Wolffian duct epithelium as well as an increase in the area of the connective tissue surrounding the ducts [32].

Concentrations of 17β-trenbolone from discharges of cattle feedlots have been reported as high as 20 ng/L [6]. These environmental concentrations are below levels that caused the adverse effects reported here for *X. tropicalis* with LOECs of 78 to 100 ng/L. These observed effects, however, illustrate the type of effects that could occur in amphibians exposed to androgenic substances in the environment. Environmental samples have been reported to possess androgenic activity based on in vitro and in vivo assays. Samples of effluent collected from concentrated cattle feedlots were reported to elicit higher responses in an androgen receptor-dependent transcriptional activation assay in CV-1 cells than 1 nM dihydrotestosterone [33]. Androgenic activity in feedlot effluent was similarly reported using the A-SCREEN bioassay, another cell-based in vitro assay [34]. Several studies have also reported field observations indicating exposure of fish to androgenic chemicals, particularly in association with kraft paper mills. Female mosquitofish (*Gambusia affinis*) collected downstream from a kraft mill effluent discharge displayed gonopodial anal fins, a male secondary sex characteristic, as well as male reproductive behaviors [35,36]. Effluent from this kraft mill also gave positive responses for androgenic activity in a variety of in vitro assays [37,38]. Broods of eelpout (*Zoarces viviparus*), a viviparous species, were found to be male biased in samples collected near kraft mills compared with reference sites [39,40].

In conclusion, 17β-trenbolone was found to alter both gonad differentiation and development of secondary sex characteristics in tadpoles of *X. tropicalis*. Presumptive females exposed to 100 ng/L trenbolone developed indications of testicular tissue within developing ovaries. The exposure concentration also resulted in premature development of nuptial pads in both sexes and hypertrophy of the Wolffian ducts. Most strikingly, this chemical elicited mortality during the later stages of tadpole development, likely via an endocrine-mediated process, for example, hypertrophy of the larynx musculature. Although measurements of trenbolone within the environment associated with cattle feedlots are lower than those that elicited responses in the present study, evidence exists for other androgenic chemicals present in the environment such as those from kraft paper mills as noted above. The results presented here suggest that amphibian species are highly sensitive to androgenic chemicals, with alterations in gonad differentiation and survival in exposed populations.

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